Vaccine 37 (2019) 7509–7518

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Combination of three virus-derived nanoparticles as a vaccine against enteric pathogens; enterovirus, norovirus and rotavirus

Suvi Heinimäki [a], Minna M. Hankaniemi [b], Amir-Babak Sioofy-Khojine [c], Olli H. Laitinen [b], Heikki Hyöty [c,d], Vesa P. Hytönen [b,d], Timo Vesikari [a], Vesna Blazevic [a,*]

[a] Vaccine Research Center, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[b] Protein Dynamics Group, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[c] Department of Virology, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
[d] Fimlab Laboratories, Tampere, Finland

ARTICLE INFO

Article history:
Received 2 May 2019
Received in revised form 23 August 2019
Accepted 20 September 2019
Available online 1 October 2019

Keywords:
Enterovirus
Coxsackievirus B
Norovirus
Rotavirus
Virus-like particle
VP6

ABSTRACT

Enteric viruses cause diverse infections with substantial morbidity and mortality in children, rotavirus (RV) and norovirus (NoV) being the leading agents of severe pediatric gastroenteritis. Coxsackie B viruses (CVB) are common enteroviruses (EV), associated with increased incidence of severe neonatal CVB disease with potentially fatal consequences. To prevent majority of childhood gastroenteritis, we have developed a non-live NoV–RV combination vaccine consisting of NoV virus-like particles (VLPs) and RV oligomeric rVP6 protein that induced protective immune responses to NoV and RV in mice. Moreover, rVP6 acted as an adjuvant for NoV VLPs. Here, we investigated a possibility to include a third enteric virus-derived antigen in the candidate NoV–RV vaccine, by adding recombinant nanoparticles derived from EV CVB1. To examine immunogenicity of EV-NoV-RV vaccine, BALB/c mice were immunized intramuscularly twice with 10 μg CVB1 VLPs, GII.4 VLPs and rVP6 nanotubes, either separately or combined. To evaluate the adjuvant effect of rVP6 on EV responses, mice received 0.3 μg CVB1 VLPs with or without 10 μg rVP6. Comparable serum IgG antibodies were detected whether the antigens were administered separately or in combination. Each formulation generated IgG1 and IgG2a antibodies, indicating a mixed Th2/Th1-type response. CVB1 VLPs skewed the isotype distribution slightly towards IgG1 subtype, while EV-NoV-RV combination vaccine induced unbiased Th1/Th2 responses to CVB1. Each antigen also induced T cell mediated immunity measured by IFN-γ secretion to specific stimulants *ex vivo*. Antisera raised by single antigens and combined formulation also exhibited strong neutralizing ability against CVB1 and NoV GII.4. Further, rVP6 showed an adjuvant effect on CVB1 responses, sparing the VLP dose and homogenizing the responses. Finally, the results support inclusion of additional antigens in the candidate NoV-RV combination vaccine to combat severe childhood infections and confirm adjuvant effect of rVP6 nanostructures.

1. Introduction

Enteric pathogens are responsible for a variety of infections with substantial morbidity and mortality rates worldwide, especially in infants and young children. Among enteric viruses, group A rotaviruses (RVs) and norovirus (NoV) are the two most medically important viruses in pediatric gastroenteritis (GE) [1]. Enteroviruses (EVs), instead, are responsible for several other commonly encountered viral infections in childhood [2].

Despite the global introduction of RV vaccinations over a decade ago and thus significant reduction in RV diarrhea-related mortality and incidence, RV still accounts for almost 40% of diarrheal hospitalizations and estimated 129,000–165,000 deaths annually in children < 5 years of age, majority of deaths taking place in low-income settings [1,3]. NoV infections have been estimated to cause about 12% of severe pediatric GE cases and 10,000–70,000 deaths among the same target population [1,4]. Despite approximately 30 divergent genotypes within the GI and GII genogroups responsible for the majority of human NoV infections [5], the GII.4 genotype has dominated since 1990s, accounting for 55–85% of all NoV GE worldwide [6]. There is no NoV vaccine available, but the current vaccine candidates rely mainly on virus-like particles (VLPs), the structures resembling the native virus particles,

* Corresponding author at: Tampere University, Vaccine Research Center, Biokatu 10, FI-33520 Tampere, Finland.
E-mail address: vesna.blazevic@tuni.fi (V. Blazevic).

https://doi.org/10.1016/j.vaccine.2019.09.072
0264-410X/

formed by spontaneous self-assembly of major capsid protein VP1 [7]. For RV, alternatives to live attenuated oral RV vaccines, including subunit vaccines, are being considered for improvement of efficacy in developing countries and alleviation of risks attributable to live vaccines [8].

Like other EVs, Coxsackievirus B (CVB) serotypes CVB1-6 cause a wide range of illnesses with clinical manifestations varying from the mild respiratory symptoms to rare but severe complications, such as aseptic meningitis and myocarditis, children under 3 years of age being the most vulnerable to the severe outcomes [2,9]. Thus far, the only licensed non-polio EV vaccines exist for EV71, but the increasing prevalence and fatality of CVB1 infections in newborns [9–11] as well as a possible contribution of CVBs to the development of type 1 diabetes [12] underscore the necessity for development of CVB vaccines. Traditional polio vaccines and recently licensed EV71 vaccines are based on live attenuated or formalin-inactivated viruses. However, to overcome the disadvantages related to the whole-virus vaccines, including reversion of the vaccine virus toward virulence, non-infectious VLPs of various EV serotypes (i.e. CVB3, EV71, CAV6, CAV16, CAV10), formed by VP0, VP1, and VP3 capsid proteins as a result of cleavage of the polyprotein P1 by viral protease, have been developed as safer vaccine candidates [13,14].

For protection against childhood GE, our laboratory has proposed a concept of vaccination against NoV and RV with a non-live subunit combination vaccine consisting of NoV GII.4 and GI.3 VLPs and RV oligomeric rVP6 protein [15,16]. RV inner capsid protein VP6 is considered a potential vaccine candidate due to its extremely immunogenic and polymorphic nature, being able to spontaneously assemble *in vitro* into diverse nanostructures, including nanotubes [17,18]. We have previously demonstrated that parenteral and mucosal administration of the candidate NoV–RV combination vaccine elicited long-lasting and broadly reactive heterologous immune responses to both NoV and RV in mice [15,16,19,20] and conferred protection against murine RV challenge [20,21]. Further, VP6 nanotubes and nanospheres have shown an adjuvant effect on immunogenicity of co-delivered NoV VLPs [22,23]. Since the number of distinct vaccines for pediatric use is constantly increasing, combination vaccines provide an effective way to combat various childhood infections with one shot. Accordingly, the present study explored the possibility of combining three different recombinant virus-derived nanoparticles, CVB1 VLPs, GII.4 VLPs and rVP6 nanotubes, into combination vaccine against EV, NoV and RV. The results demonstrate that no immunological interference exists between the three tested vaccine antigens.

2. Materials and methods

2.1. Production of antigens

EV CVB1 VLPs [24], NoV GII.4 VLPs and RV rVP6 proteins were produced in baculovirus (BV)-insect cell expression systems, as described in detail elsewhere [15,25,26]. CVB1 VLPs were purified according to a recently developed protocol consisting of Tangential Flow Filtration and multistep chromatographic procedures [24], a modification of the method originally developed for CVB3 VLPs [13]. GII.4 VLPs were purified with polyethylene glycol precipitation followed by anion exchange chromatography [26,27]. RV rVP6 was purified by sucrose gradients and ultracentrifugation [15,25] followed by three consecutive ultrafiltration procedures [18].

2.2. Characterization of vaccine antigens

The purity, identity and morphology of all three vaccine antigens were determined using previously described procedures [18,22]. Shortly, the absence of BV was verified by a BacPAK Rapid-Titer Kit (Takara, Cat. 631406) and SDS-PAGE followed by immunoblotting with anti-BV gp64 antibody (Santa Cruz Biotechnology, Inc., Cat. sc-65499). Endotoxin levels were determined using ToxinSensor™ Gel Clot Endotoxin Assay kit (GenScript, Cat. L00351) according to manufacturer's instructions.

The integrity and morphology of the protein assemblies were examined by transmission electron microscope (TEM) with Jeol JEM-1400 (Jeol Ltd., Tokyo, Japan) following negative staining with 3% uranyl acetate (pH 4.6). Hydrodynamic diameter (size), volume distribution (%) and polydispersity index (PdI) of the antigens were determined by dynamic light-scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK) equipped with a He–Ne laser (633 nm).

2.3. Animal immunization and sample collection

Female 6-week-old BALB/c OlaHsd mice (Envigo, Horst, the Netherlands) were randomly divided into seven groups (Gr I-VII), acclimatized under controlled specific pathogen-free conditions for one week prior to the start of the experiment, and maintained throughout the study period with food and water provided ab libitum. Animals were immunized twice (at study weeks 0 and 3) intramuscularly at the right caudal thigh muscle with 50 μl of CVB1 VLPs, GII.4 VLPs, rVP6 protein, a mixture of these three antigens or a mixture of CVB1 VLPs and rVP6 (Table 1). To assess immunogenicity induced with EV, NoV, and RV antigen alone or in the mixture, the dose of each immunogen was 10 μg per immunization point. To evaluate the adjuvant effect of VP6 on CVB1 induced response, two groups of mice received suboptimal 0.3 μg dose of CVB1 VLPs alone or in a combination with 10 μg VP6. No external adjuvants were included in any vaccine formulation. Control group received carrier only (sterile phosphate buffered saline, PBS). Immunizations were conducted under general anesthesia by inhalation of isoflurane (Attane vet, Vet Medic Animal Health Oy Cat. AP/DRUGS/220/96).

Blood samples were taken before each immunization (study weeks 0 and 3) by tail bleeding to test for the kinetics of the serum antibody responses. Whole blood, feces, nasal washes (NWs) and splenocytes were collected at the time of sacrifice (study week 5) and processed according to the previously published procedures [15,19,28]. Each experimental procedure was carried out in accordance with the regulations and guidelines of the Finnish National Experiment Board (Permission number ESAVI/10800/04.10.07/2016). All efforts were made to minimize animal suffering and to reduce the number of animals used. Animal health was monitored throughout the experiment.

2.4. Antigen–specific serum and mucosal antibody responses

Serum samples of individual mice were tested in ELISA for the presence of EV CVB1-, NoV GII.4- and RV VP6-specific IgG and IgG subtype antibodies as described elsewhere [15,28]. Further,

Table 1
Antigenic formulations and immunization of experimental mice.

Experimental group	Immunogen	Injection dose (μg)	# mice/group
I	CVB1 VLP	0.3	5
II	CVB1 VLP	10	5
III	GII.4 VLP	10	3
IV	VP6	10	3
V	CVB1 VLP + GII.4 VLP + VP6	10 + 10 + 10	5
VI	CVB1 VLP + VP6	0.3 + 10	5
VII	PBS (Control)	–	5

10% fecal suspensions and NWs were studied for anti-CVB1 IgG antibodies. Shortly, 96-well half-area polystyrene plates (Corning Inc., Cat. 3690) were coated with 50 ng of CVB1 VLPs, GII.4 VLPs or rVP6 per well. Antigen-specific antibodies in sera or mucosal secretions were detected with horseradish peroxidase (HRP) -conjugated anti-mouse IgG (Sigma-Aldrich, Cat. A4416), IgG1 (Invitrogen, Cat. A10551) or IgG2a (Invitrogen, Cat. A10685) and SIGMA FAST OPD substrate (Sigma-Aldrich, Cat. P9187). Optical densities at 490 nm (OD_{490}) were measured by Victor^2 microplate reader (PerkinElmer, Waltham, MA). Endpoint titers were expressed as the reciprocal of the highest sample dilution with an OD_{490} above the cut-off value (>0.1 OD_{490} unit).

2.5. Neutralizing anti-CVB1 antibody detection

Neutralizing ability of immune sera against CVB1 (strain CVB1-V200, kindly provided by Vactech Ltd.) was measured in fourfold serially diluted sera by standard virus plaque reduction assay in green monkey kidney (GMK) cells as previously described [12]. A reduction in plaque number $\geq$ 80% compared with mock-treated virus control was considered positive. A titer of 16 was assigned for positivity threshold of neutralizing capacity.

2.6. NoV blocking assay

The ability of serum antibodies to block NoV VLP binding to a cellular histo-blood group antigen (HBGA) receptors was tested with NoV blocking assay, utilizing pig gastric mucin (PGM) type III (Sigma Chemicals, Cat. M1778) as a source of HBGAs [29] according to the recently described procedure [23]. In brief, mixtures of pre-incubated GII.4 VLPs and serially diluted sera were added on PGM coated 96-microwell plates and the bound VLPs were detected with human GII.4 antiserum followed by the corresponding HRP-conjugated secondary IgG (Novex, Cat. A18811). The blocking titer 50 (BT_{50}) was determined as the reciprocal of the final serum dilution blocking $\geq$50% of the maximum VLP binding determined with VLPs without serum.

Table 2
Specifications of vaccine antigens.

Specification	CVB1 VLPs	GII.4 VLPs	rVP6
Identity	VP0, VP1 and VP3	VP1 doublet	VP6
Morphology	VLPs (~30 nm)	VLPs (~37 nm)	Tubes (~0.2–1.5 μm)
Size (d.nm)*	30.36 ± 0.20	36.60 ± 0.45	672.86 ± 57.62
Infectious BV (pfu/ml)	0	0	0
BV gp64	Negative	Negative	Negative
Endotoxin (EU/10 μg protein)	<0.06	<0.02	<0.01

* Average of three measurements (each measurement containing 10–20 × 10 s datasets at 25 °C) ± standard deviation.

2.7. Cell-mediated immune response detection

T cell responses were quantified using an ELISPOT IFN-γ assay previously published by our laboratory [16,25]. For detection of CVB1-, GII.4-, and VP6-specific IFN-γ producing cells, liquid nitrogen frozen splenocytes (0.2×10^6 cells/well) were stimulated in duplicates with CVB1 or GII.4 VLPs (20 μg/ml) or an 18-mer VP6-derived R6-2 peptide (5 μg/ml) previously identified as a VP6-specific CD4$^+$ T cell epitope (^{242}DGATTWYFNPVILRPNNV^{259}) [30], respectively. Background control (culture medium) and cell viability control (T cell mitogen Concanavalin A) were tested in each assay. The spots representing individual IFN-γ secreting cells were counted by ImmunoSpot® automatic CTL analyzer (CTL-Europe GmbH, Bonn, Germany). The results were expressed as mean spot-forming cells (SFC)/10^6 splenocytes of duplicate wells.

2.8. Statistical analyses

The statistical differences between the non-parametric observations of two or more independent groups were determined with the Mann-Whitney *U* test and Kruskal-Wallis tests. Data was analyzed with GraphPad Prism software, version 8.0.1, and $p < 0.05$ was defined to indicate statistically significant difference.

3. Results

3.1. Characterization of vaccine antigens

Purity, integrity and morphology of CVB1 VLPs, GII.4 VLPs and rVP6 protein were verified as shown in Table 2. SDS–PAGE analysis showed presence of EV CVB1 capsid proteins VP0, VP1 and VP3

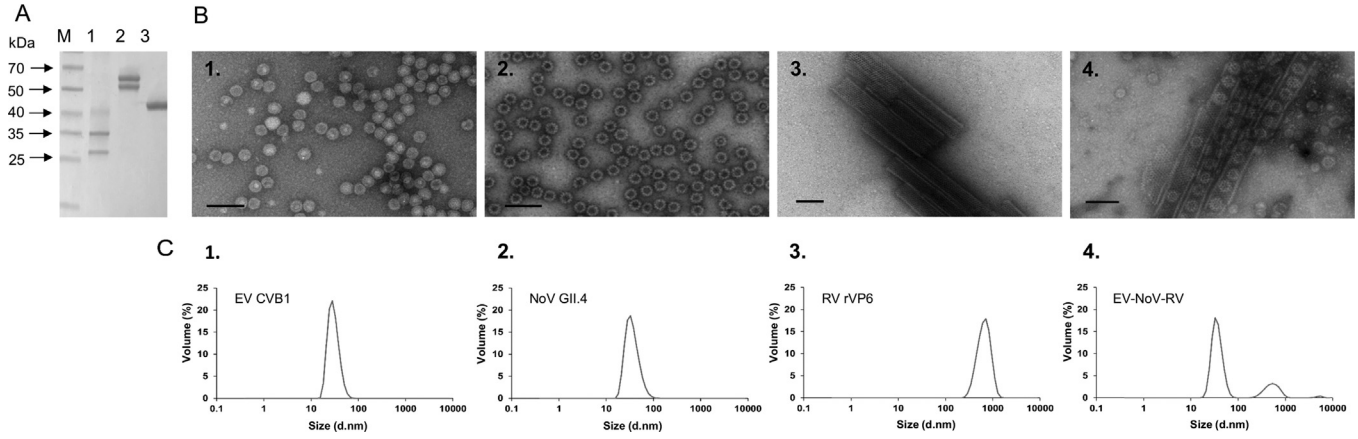

Fig. 1. Characterization of the vaccine antigens. (A) SDS-PAGE analysis of purified EV CVB1 VLPs (lane 1), NoV GII.4 VLPs (lane 2), and RV rVP6 protein (lane 3). Lane M illustrates molecular weight marker. (B) Electron micrographs of morphological structures assembled by EV CVB1 VLPs (panel 1), NoV GII.4 VLPs (panel 2), RV rVP6 nanostructures (panel 3), and a mixture (1:1:1) of aforementioned antigens (EV-NoV-RV combination vaccine, panel 4) corresponding to SDS-PAGE lanes 1–3, respectively. Black bar indicates 100 nm. (C) Dynamic light scattering (DLS) analysis of the vaccine antigens. Size distributions in nanometers (nm) by volume percent of EV CVB1 VLPs (panel 1), NoV GII.4 VLPs (panel 2), RV rVP6 nanotubes (panel 3), and a mixture (1:1:1) of aforementioned antigens (EV-NoV-RV combination vaccine, panel 4) are shown. The results are presented as the average of three measurements (each measurement containing 10–20 × 10 s datasets at 25 °C).

[13], NoV GII.4 VP1 doublet [26], and RV VP6 protein bands [25] (Fig. 1A). No residual impurities were detected in any of the purified proteins, as antigens were free of live BV as well as BV gp64 protein (Table 2). Further, none of the antigenic formulations contained bacterial endotoxins in excess levels [31] (Table 2). Each antigen preparation consisted of particles of the expected size and morphology, including CVB1 VLPs, GII.4 VLPs and rVP6 nanotubes, confirmed under TEM (Fig. 1B, Table 2). Mixing the CVB1 VLPs, GII.4 VLPs and rVP6 in equal quantities did not impair single protein integrity or morphology (Fig. 1B).

Analysis of antigenic formulations by DLS showed that 100% of the CVB1 VLP, GII.4 VLP and rVP6 nanotubes had respective average hydrodynamic diameters of 30 nm (PdI 0.05), 37 nm (PdI 0.10), and 673 nm (PdI 0.25) (Fig. 1C), corresponding to sizes determined by TEM (Table 2). Only two distinct populations were observed in the combined vaccine formulation (PdI 0.78), where CVB1 and GII.4 VLPs with close particle sizes were recognized as the one single particle population (79%) and rVP6 nanotubes as the distinct population (21%) (Fig. 1C).

3.2. No mutual inhibition of vaccine antigens

3.2.1. Induction of robust IgG response

To examine immunogenicity induced with EV-NoV-RV combination vaccine and possible mutual interference of the vaccine components with immunogenicity, vaccine antigens were administered either separately or in combination at 10 μg doses/antigen. Each vaccine antigen elicited a robust IgG response (geometric mean titers, GMTs $\geq$ 4.4 Log_{10}) (Fig. 2). No statistically significant differences between the magnitudes of IgG antibody levels ($p \geq 0.09$) were detected whether CVB1 VLPs (Fig. 2A), GII.4 VLPs (Fig. 2B) or rVP6 (Fig. 2C) were administered alone or in the trivalent combination. Sera from control mice did not show reactivity with any of the antigens (Fig. 2).

3.2.2. Induction of mixed Th2/Th1 responses

Determination of antigen-specific IgG subtype IgG1 and IgG2a titers, representing Th2- and Th1-type responses, revealed induction of IgG1 (GMTs $\geq$ 4 Log_{10}) and IgG2a (GMTs $\geq$ 3.4 Log_{10}) antibodies with each antigenic formulation (Fig. 3). CVB1 VLPs alone generated significantly higher ($p = 0.018$) levels of IgG1 (GMT 5.5 Log_{10}) compared with the levels induced by the combination (GMT 4.6 Log_{10}) (Fig. 3A). By contrast, combined formulation caused threefold greater anti-CVB1 IgG2a titers (GMT 4.7 Log_{10}) than single antigen (GMT 4.2 Log_{10}); however, the difference was not significant ($p = 0.331$) (Fig. 3B). Thus, CVB1 VLPs skewed the response slightly towards Th2-type, while the administration in combination with GII.4 VLPs and rVP6 resulted in better-balanced Th2/Th1 response. Comparable GII.4-specific IgG1 ($p = 0.237$) and IgG2a ($p = 0.877$) levels were elicited, whether GII.4 VLPs were administered separately or in the combination with other antigens (Fig. 3C and D). Similarly, no significant difference was observed in the magnitude of VP6-specific IgG1 ($p = 0.127$) or IgG2a ($p = 0.365$) responses between rVP6 alone and combined formulation (Fig. 3E and F). No antigen-specific IgG subtype antibodies were detected in sera of control mice (Fig. 3).

3.2.3. Induction of CVB1- and GII.4-specific neutralizing antibodies

Ability of induced antibodies to neutralize the virus was studied as a protective potential of antisera. Fig. 4A depicts ability of serum antibodies to neutralize CVB1. Immunization with CVB1 VLPs alone conferred similar neutralization (GMT 446) to the combination vaccine (GMT 588) ($p = 0.738$). Sera from control mice did not exhibit neutralization activity at 1:16, the lowest dilution tested (Fig. 4A).

Neutralizing ability of GII.4-specific antibodies was examined measuring the blocking of GII.4 VLPs binding to the HBGAs. Both

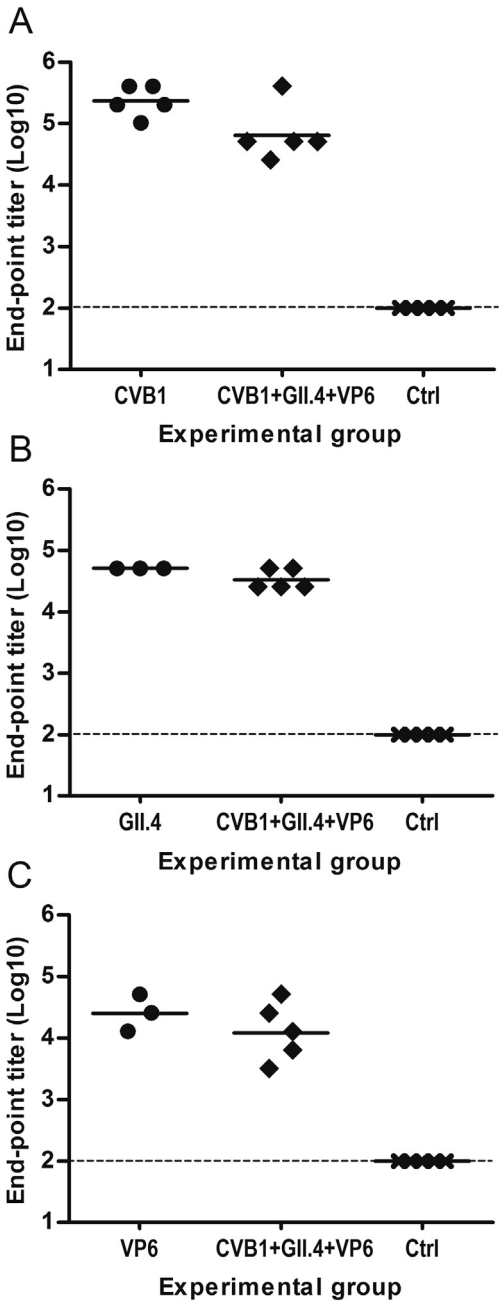

Fig. 2. Antigen-specific serum IgG antibody responses following immunization with 10 μg doses of EV CVB1 VLPs, GII.4 VLPs, or RV rVP6 or a mixture of the three antigens. Shown are CVB1- (A), GII.4- (B), and VP6-specific (C) end-point titers of IgG antibodies in termination sera at week 5. Each symbol represents an individual mouse. Control (Ctrl) mice received PBS only. The solid line indicates the geometric mean titer of the group. A titer of 100 was assigned for sera with no detectable antibodies, being a half of the initial dilution. Horizontal dashed line indicates the cut-off level (2 Log_{10}).

GII.4 VLP formulations induced antibodies able to block efficiently the VLP binding (Fig. 4B). Blocking activity was comparable ($p = 0.696$) whether GII.4 VLPs were administered separately (BT_{50} 400 ± 200) or in the trivalent combination (BT_{50} 240 ± 40). Control mice did not induce blocking antibodies (Fig. 4B).

3.2.4. Induction of antigen-specific IFN-γ secreting T cells

Induction of T cell responses by antigenic formulations was further characterized measuring Th1-type cytokine IFN-γ production from the splenocytes of immunized and control mice. Each vaccine antigen elicited a considerable T cell response to *ex vivo* stimula-

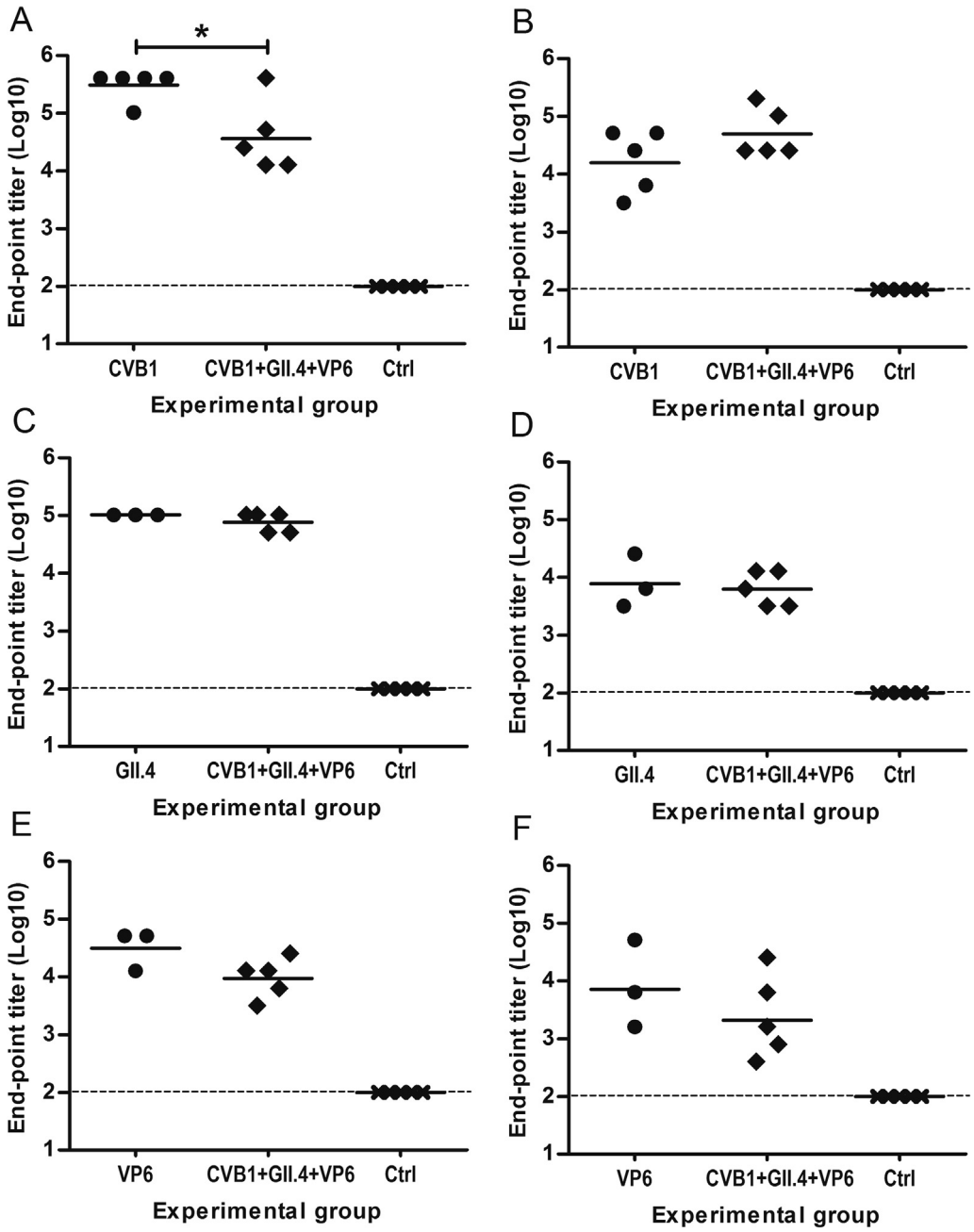

Fig. 3. Antigen-specific serum IgG subtype antibody responses following immunization with 10 μg doses of EV CVB1 VLPs, GII.4 VLPs, or RV rVP6 or a mixture of the three antigens. Shown are CVB1- (A, B), GII.4- (C, D), and VP6-specific (E, F) end-point titers of IgG1 (A, C, E) and IgG2a (B, D, F) antibodies in termination sera at week 5. Each symbol represents an individual mouse. Control (Ctrl) mice received PBS only. The solid line indicates the geometric mean titer of the group. A titer of 100 was assigned for sera with no detectable antibodies, being a half of the initial dilution. Horizontal dashed line indicates the cut-off level (2 Log_{10}).

tion with CVB1 or GII.4 VLPs or the R6-2 peptide (Fig. 5). Similar quantities of IFN-γ secreting cells ($p \geq 0.4$) were detected whether CVB1 VLPs (Fig. 5A), GII.4 VLPs (Fig. 5B) or rVP6 (Fig. 5C) were administered alone or in the trivalent combination. No antigen-specific IFN-γ responses were developed by the cells of negative control mice (Fig. 5A–C). Culture media alone stimulated no IFN-γ production by the cells from any of the groups (Fig. 5A–C).

3.3. VP6 effect on immunogenicity of CVB1 VLPs

3.3.1. Development of CVB1-specific serum antibodies

An adjuvant effect of rVP6 on CVB1-specific antibody responses was investigated immunizing mice twice with 0.3 μg of CVB1 VLPs alone or together with 10 μg of rVP6. Moreover, results of mice immunized with 10 μg dose of CVB1 VLPs were included for comparison. Fig. 6A depicts development of serum anti-CVB1 IgG antibodies at weeks 0, 3 and 5. Three weeks after the first dose, IgG was present in all experimental groups. However, the first 0.3 μg dose of CVB1 VLPs alone induced only minor IgG response (OD_{490} 0.243 ± 0.139), antibody levels being significantly lower ($p = 0.039$) than the levels induced by 10 μg of CVB1 VLPs (OD_{490} 1.108 ± 0.275) and co-administration of 0.3 μg with rVP6 (OD_{490} 0.752 ± 0.152) (Fig. 6A). Instead, co-delivery of CVB1 VLPs with rVP6 evoked equally high ($p = 0.347$) IgG response with 10 μg dose of VLPs alone. No significant differences in the responses were observed after the second dose administration, given at week 3,

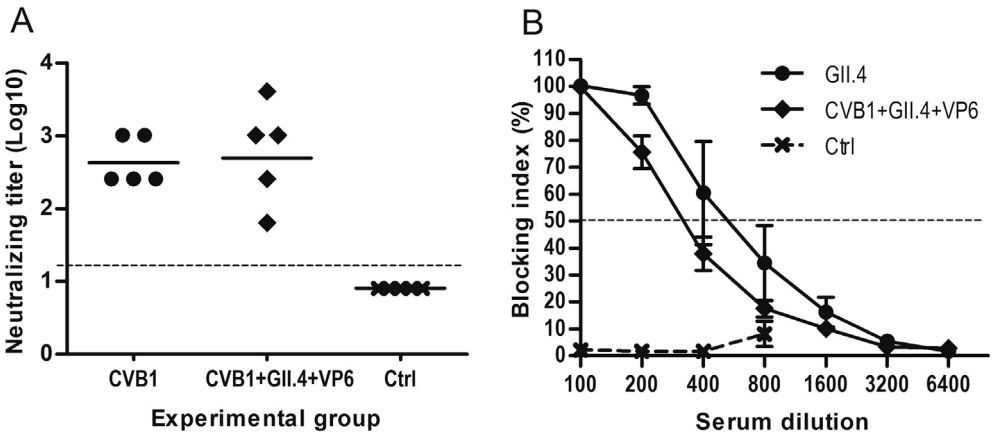

Fig. 4. Neutralizing antibodies induced by 10 μg of EV CVB1 or GII.4 VLPs, or a trivalent EV-NoV-RV combination consisting of CVB1 VLPs, GII.4 VLPs and rVP6 nanostructures. Control (Ctrl) mice received PBS only. (A) Neutralizing antibody titers against CVB1. Each symbol represents an individual mouse. The solid line indicates the geometric mean titer of the group. A titer of 8 was assigned for all serum samples with no detectable neutralizing antibodies, being a half of the initial dilution. Horizontal dashed line indicates the positivity threshold of neutralizing capacity (1.2 Log_{10}). (B) Homologous blocking of GII.4 VLP binding to histo-blood group antigens. The results are expressed as the mean blocking indexes (%) of experimental groups (±SEM), calculated as follows: 100% - OD_{490} ((wells with serum) / OD_{490} (wells without serum) × 100%). The horizontal dashed line represents the blocking titer 50% (BT_{50}).

for any of the experimental groups ($p = 0.613$). Control mice remained negative for antigen-specific IgG response during the whole study period (Fig. 6A).

CVB1-specific serum IgG, IgG1 and IgG2a antibody titers of immunized mice are shown in Fig. 6B–D. Regardless of lack of significance in the magnitudes of IgG ($p = 0.196$) (Fig. 6B) or IgG1 ($p = 0.289$) (Fig. 6C), mice receiving 0.3 μg dose of CVB1 VLPs in combination with rVP6 developed tenfold greater and more uniform responses than the mice receiving 0.3 μg dose of CVB1 VLPs alone. Similarly, addition of rVP6 in the CVB1 VLP formulation increased IgG2a titers fourfold, although the difference was statistically insignificant ($p = 0.398$) (Fig. 6D).

3.3.2. Development of serum neutralizing antibodies

Irrespective of CVB1 VLP formulation, all experimental groups mounted a considerable neutralization activity of CVB1 (Fig. 6E). No statistical difference in anti-CVB1 neutralizing titers ($p = 0.915$) was observed between the mice receiving 0.3 μg of CVB1 VLPs alone (GMT 256) or combined with 10 μg of rVP6 (GMT 339), but the neutralizing titers were somewhat more evenly distributed after inclusion of rVP6. These neutralizing antibody responses were comparable ($p = 0.169$) with the response induced by 10 μg dose of CVB1 (GMT 446).

3.3.3. Increase in anti-CVB1 cellular immunity

Irrespective of CVB1 VLP formulation, splenocytes from immunized mice responded with considerable IFN-γ release, when stimulated with CVB1 VLPs (Fig. 6F). Regardless of a lack of significance in the magnitudes of IFN-γ responses ($p = 0.057$), mice receiving 0.3 μg dose of CVB1 VLPs in combination with rVP6 developed twofold greater quantities of IFN-γ secreting cells than the mice receiving 0.3 μg dose of CVB1 VLPs alone. The T cell responses were comparable ($p = 0.4$) with the response induced by 10 μg dose of CVB1.

3.3.4. Induction of anti-CVB1 antibodies in mucosal secretions

To investigate if rVP6 has an influence on stimulation of antibodies at mucosal surfaces, group-wise pooled 10% fecal suspensions and individual NW specimens were tested for the presence of anti-CVB1 IgG. Comparison of fecal responses (Fig. 7A) indicated that 0.3 μg dose of CVB1 VLPs induced twofold lower levels of intestinal IgG (endpoint titer 10) than 10 μg dose or 0.3 μg dose co-administered with rVP6 (endpoint titers 20). As expected, antibodies in nasal secretions (Fig. 7B) showed similar pattern to that observed in feces. Of mice immunized with 0.3 μg dose of VLPs in the absence of rVP6, only 1/5 had low level of nasal IgG (GMT 3.3). In comparison, nasal lavages showed fourfold increase in mucosal IgG levels ($p = 0.109$), when mice received either 10 μg dose (GMT 15.2) or 0.3 μg dose co-administered with rVP6 (GMT 13.2), but the levels varied among individual mice. Control mice had no detectable mucosal anti-CVB1 IgG antibodies (Fig. 7).

4. Discussion

Combination vaccines are the cornerstones for public health benefits, reducing the number of required injections while increasing immunization compliance. Incorporation of diphtheria, pertussis and tetanus antigens into a single vaccine formulation represents the first successful example of combination vaccines [32]. Due to the high global social and economic burden caused by RV and NoV infections [1], our laboratory has recently developed a combined non-live subunit vaccine against these two frequent causes of childhood GE [15,16]. The candidate vaccine consisting of NoV GII.4 and GI.3 VLPs and RV rVP6 protein was highly immunogenic in mice by inducing protective immune responses against NoV and RV [15,16,21]. To combat many common enteric childhood infections with the same vaccine, this study was aimed to investigate possibility to include EV antigens in the candidate NoV–RV combination vaccine. Based on the increased incidence of severe CVB disease with potentially fatal cardiological and neurological involvement [9–11], we selected CVB1 as a model pathogen and generated EV-NoV-RV combination vaccine comprising EV CVB1 VLPs, NoV GII.4 VLPs and RV rVP6 nanotubes. To exclude a potential immunological interference of distinct antigenic components [32], we compared antigen-specific immunogenicity raised by individual antigens and EV-NoV-RV combination vaccine. While comprehensive preclinical studies with the NoV–RV vaccine have already demonstrated no mutual interference and/or inhibition of NoV GII.4 VLPs, NoV GI.3 VLPs, and RV rVP6 with antibody and T cell responses [15,16], the present study indicates unaltered GII.4- and VP6-specific systemic antibody and T cell responses by CVB1 VLPs. Thus, this data supports the possibility to add EV (CVB) antigens to NoV–RV candidate vaccine.

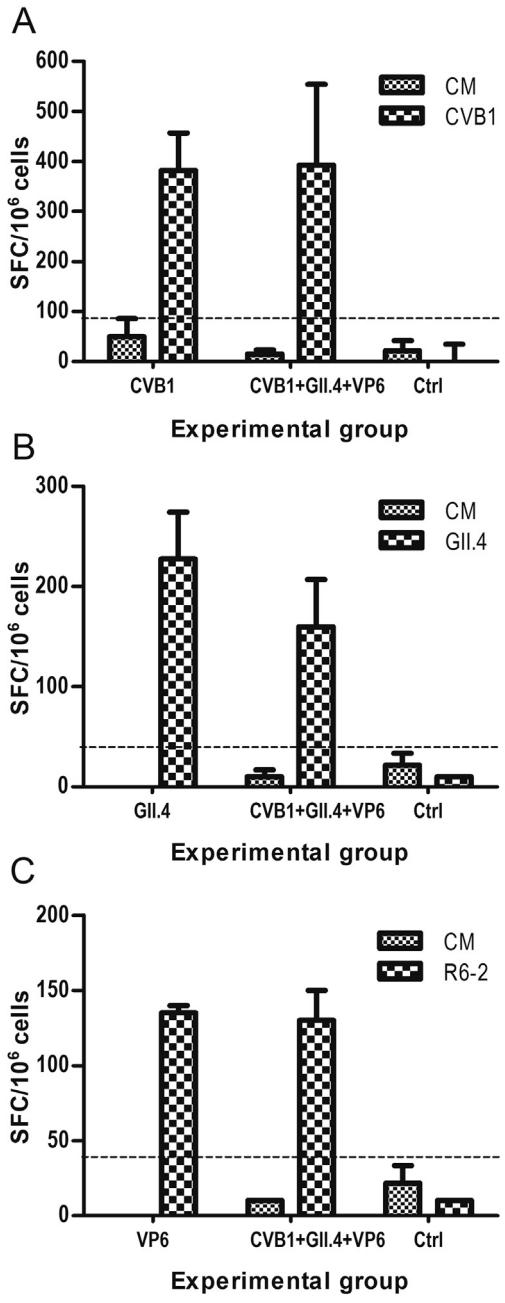

Fig. 5. Antigen-specific T cell responses following immunization with 10 μg doses of EV CVB1 VLPs, GII.4 VLPs, or RV rVP6 or a mixture of the three antigens. Shown are CVB1- (A), GII.4- (B), and VP6-specific (C) IFN-γ responses following stimulation of splenocytes with CVB1 or GII.4 VLPs or VP6-specific R6-2 peptide. Control (Ctrl) mice received PBS only. Results are expressed as mean IFN-γ spot forming cells (SFC)/10^6 cells of experimental groups with standard error of the means. The dashed lines indicate the cut-off limit obtained from cells incubated in a culture media (CM) only (mean SFC/10^6 + 3 × SD).

Induction of strong systemic antigen-specific antibody responses by CVB1 VLPs, GII.4 VLPs and rVP6 nanotubes demonstrated high immunogenicity of all antigens. Serum IgG levels were comparable whether the antigens were administered separately or in the trivalent combination, suggesting immunological compatibility of the three antigens. Further, analysis of IgG subclasses as markers of cellular responses revealed that each antigenic formulation generated IgG1 and IgG2a responses, indicating a mixed antigen-specific Th2/Th1-type response. Induction of cellular immunity by each antigenic formulation was confirmed measuring antigen-specific IFN-γ cytokine production, a hallmark of Th1 cell immunity. The multivalent antigen expression, size and morphology of CVB1 VLPs, GII.4 VLPs and rVP6 nanotubes make these antigens potent inducers of antibodies and T cell responses, through efficient cross-linking of B cell receptors [33] and internalization by antigen-presenting cells (APCs) [34]. Observed responses are consistent with induction of both Th1 and Th2 cells previously seen with delivery of NoV VLPs, RV rVP6 as well as NoV–RV combination vaccine [15,16]. Administration of CVB1 VLPs alone skewed the isotype distribution towards IgG1 subtype, whereas combination vaccine promoted unbiased Th1/Th2 responses to CVB1. In concordance with the immunity induced by CVB1 VLP formulations, EV71 VLPs have also been reported to stimulate a mixed Th1- and Th2-type response in IM immunized mice [35].

Although the role of cell-mediated immune responses in the EV clearance and protection is less characterized, $CD4^+$ and $CD8^+$ T cell responses, in addition to neutralizing antibodies, have been demonstrated to be involved in immunity induced by oral poliovirus vaccine [36]. Similarly, T cell immunity appears to have a role in the generation of heterologous NoV immunity [37,38]. In contrast to EV and NoV VLPs derived from viral capsid protein/s, RV inner capsid VP6 protein does not elicit classical neutralizing antibodies, but pIgR-mediated intracellular neutralization and $CD4^+$ T cells are considered the principal mediators of VP6-inducecd protection [39,40]. Indeed, we have recently demonstrated an ability of rVP6 nanostructures to induce Th1, Th2 and Th17 cell subsets as well as $CD4^+$ cytotoxic T lymphocytes with the potential to lyse RV infected cells [41].

To explore functionality of induced immunity, protective potential of antibodies in terms of neutralization was confirmed. Antisera raised by CVB1 VLPs, either separately or in trivalent combination, exhibited a strong neutralizing capacity against the homologous CVB1 strain *in vitro*. This is in agreement with the robust IgG1 responses, which have been associated with EV neutralization [42]. Neutralization titers were comparable with those induced previously with inactivated CVB1 vaccine [43] as well as CVB3 VLPs [13]. In support of good compatibility of CVB1, GII.4 and rVP6 antigens, Wang and colleagues [44] have observed no interference between EV71 and GII.4 VLPs with respect to their protective potential.

Despite the recent progress in cultivation of NoV *in vitro*, the blocking assay is still employed as a surrogate measure for NoV neutralization. NoV-specific antibodies, which block the binding of NoV VLP to its putative HBGA receptors or attachment factors, are considered the best correlate of protection against NoV infection [45–47]. The data in this study demonstrated that inclusion of CVB1 VLPs in the candidate NoV–RV vaccine formulation did not alter the blocking potential of anti-GII.4 antibodies.

We have previously reported rVP6 oligomers to have adjuvant properties, promoting adaptive immune responses against co-delivered NoV VLPs [23]. rVP6 was shown to improve responses to the foreign antigenic peptides, when employed as an immunological carrier [48]. Thus, this study was extended to investigate adjuvant effect of rVP6 on immunogenicity of CVB1 VLPs, employing the suboptimal 0.3 μg dose of CVB1 VLPs according to our previous studies with NoV VLPs [22,23]. In here, the suboptimal dose of CVB1 VLPs induced unexpectedly high if variable responses, hampering to address an evident adjuvant action of rVP6. However, the addition of rVP6 did result in constantly more uniform CVB1-specific responses, referring to the stabilizing effect of rVP6 in the formulation. Although rVP6 nanostructures were shown to improve the blocking potential of NoV-specific antibodies [22,23], the similar effect of rVP6 could not be observed on protective CVB1-specific responses, possible due to the considerable immunogenicity of CVB1 VLPs at the selected dose. However, the more consistent neutralization titers mirrored the more consistent

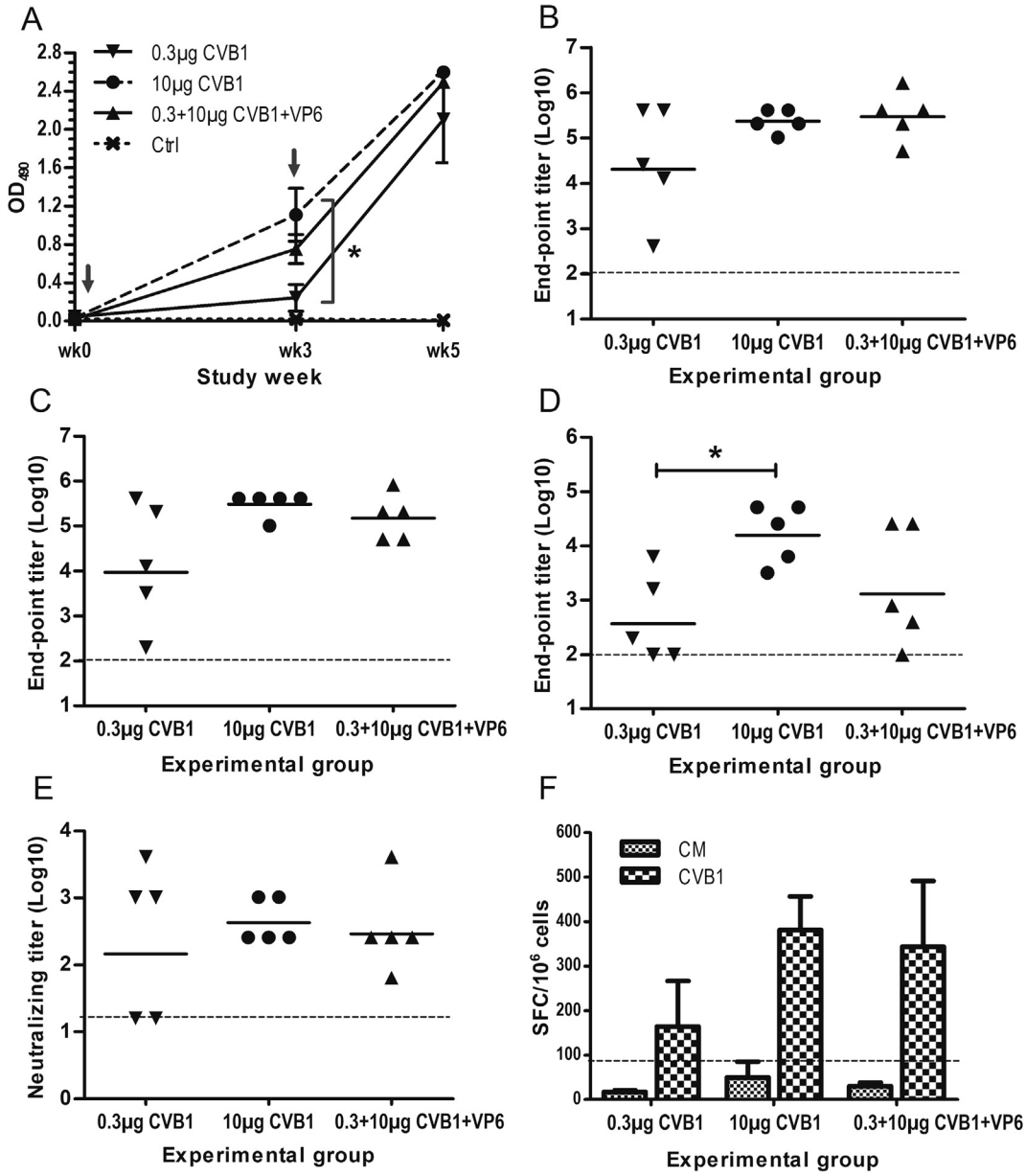

Fig. 6. Development of EV CVB1-specific antibodies and T cell responses following immunization with 0.3 µg or 10 µg of CVB1 VLPs alone or 0.3 µg dose formulated with 10 µg of rVP6. Control (Ctrl) mice received PBS only. (A) Kinetics of total IgG antibodies in sera. Group mean OD_{490} values with standard error of means of 1:200 diluted sera at indicated study weeks are shown. Immunizations at study weeks 0 and 3 are shown with arrows. Horizontal dashed line indicates the cut-off level ($OD_{490} \geq 0.1$). End-point titers of IgG (B), IgG1 (C), and IgG2a (D) antibodies in termination sera at week 5. Each symbol represents an individual mouse. The solid line indicates the geometric mean titer of the group. A titer of 100 was assigned for sera with no detectable antibodies, being a half of the initial dilution. Horizontal dashed line indicates the cut-off level (2 Log_{10}). (E) Protective potential of anti-CVB1 antibodies. Shown are neutralizing antibody titers against CVB1, each symbol representing an individual mouse. The solid line indicates the geometric mean titer of the group. A titer of 8 was assigned for all serum samples with no detectable neutralizing antibodies, being a half of the initial dilution. Horizontal dashed line indicates the positivity threshold of neutralizing capacity (1.2 Log_{10}). (F) IFN-γ production by T cells. Splenocytes from immunized mice were simulated *ex vivo* with CVB1 VLPs. Results are expressed as mean IFN-γ spot forming cells (SFC)/10^6 cells of experimental groups with standard error of the means. The dashed line indicates the cut-off limit obtained from cells incubated in a culture media (CM) only (mean SFC/10^6 + 3 × SD).

IgG1 responses. To clarify the extent of rVP6 action more evidently, the experimental design with a lower dose of CVB1 VLPs needs to be accomplished. Nevertheless, the adjuvant effect of VP6 on NoV-specific immune responses has been elucidated to be dependent on co-delivery and co-localization of the antigens [22,23]. Accordingly, RV VP6 upregulates expression of antigen presentation molecules, co-stimulatory molecules, and pro-inflammatory cytokines on APCs [49], creating a favorable environment for uptake and presentation of co-delivered antigens.

In conclusion, our data shows that neither NoV GII.4 VLPs nor RV rVP6 nanotubes interfered with EV CVB1-specific antibody levels or vice versa. Therefore, it may be possible to include CVB1 VLPs, and possibly other EV antigens, in the NoV–RV candidate vaccine formulation in order to generate vaccine against divergent enteric infections. The current study supports our previous observation that rVP6 nanostructures have desirable features attributed to classical adjuvants [22,23], which function to spare the dose of the antigens and accelerate and improve the immune responses. Thus, rVP6 nanotubes do not only act as an adjuvant for NoV VLPs but they also exert an adjuvant effect on CVB1-specific responses. Overall, these findings further support the use of RV VP6 protein in combined vaccines against enteric pathogens.

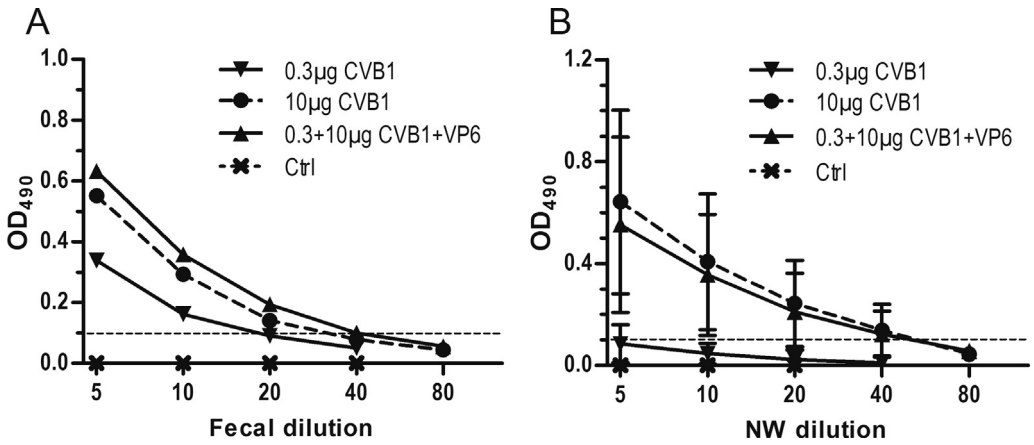

Fig. 7. Mucosal CVB1-specific antibody responses following immunization with 0.3 μg or 10 μg of CVB1 VLPs alone or 0.3 μg dose formulated with 10 μg of rVP6. End-point titrations of anti-CVB1 IgG antibodies in 10% fecal suspensions (A) or nasal washes (NWs) (B) of experimental mice. Control (Ctrl) mice received PBS only. Mean titration curves with standard errors of the mean of the experimental groups are shown. Horizontal dashed line indicates the cut-off level ($OD_{490} \geq 0.1$).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: H.H. is a minor (5%) shareholder and member of the board of Vactech Ltd, which develops vaccines against picornaviruses. H. H. serves on the scientific advisory board of Provention Bio Inc., which is developing an enterovirus vaccine. The other authors have no conflict of interest to declare.

Acknowledgements

We gratefully acknowledge the Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki. Special thanks are due to Dr. Helena Vihinen and Mervi Lindman for the guidance and technical help in transmission electron microscopy. The technical assistance given by the personnel of Vaccine Research Center and Ulla Kiiskinen from Protein Dynamics group of Tampere University is acknowledged. We also express our gratitude to members of the animal facility at Tampere University. We acknowledge Business Finland (project THERDIAB 1843/31/2014) for financial support and the partners of the THERDIAB project (ArcDia Ltd., Vactech Ltd., JILab Ltd., FimLab Ltd.) for their support. Finally, we acknowledge Biocenter Finland for infrastructure support.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.09.072.

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